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Thyroid hormone (T3) is essential for normal skeletal growth and development. Excess T3, especially in adult individuals, can lead to bone loss and increased susceptibility to fractures. Data from our laboratory showed that in in vitro systems, T3 increases the local production of both a critical bone growth factor, insulin-like growth factor-I. It also potentiated interleukin-1 (IL-1) stimulated production of a cytokine with significant importance for osteoclastogenesis, interleukin-6 (IL-6). The current studies were undertaken to determine whether these local factors are important in the effects of T3 to stimulate osteoblast proliferation and to generate bone-phenotypic proteins, i.e., alkaline phosphatase, osteocalcin, collagen and in the effects of T3 to stimulate bone resorption. The data obtained supports these conclusions, as antibody, antisense or antagonist peptide to the IGF-I receptor inhibited the anabolic effects of T3 and antibody to the IL-6 receptor inhibited the bone resorbing effect of T3. Also, studies were undertaken to investigate the mechanisms by which T3 stimulates the production of insulin-like growth factor-I and interleukin-6. IL-1 increased IL-6 mRNA and IL-6 promoter construct expression, and in some studies T3 had small stimulatory effects and enhanced the response to IL-1. The studies on the mechanism of the thyroid effect on IGF-I synthesis have not yet provided any conclusive results and are still ongoing.

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Paula H. Stern 10/21/99

PI - Signature Date

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INTRODUCTION

The **subject** and **purpose** of our study was to determine the mechanisms by which excess thyroid hormone (T3) causes bone loss and increased susceptibility to fractures. This is an important problem since hyperthyroidism is a relatively common disease and also because thyroid hormones are a frequently prescribed medication, both appropriately and inappropriately. Also, there is the potential for excessive thyroid hormone intake through non-proprietary remedies. Although this has been more tightly regulated during recent years, such products are still available. T3-mediated bone loss represents a particularly interesting problem because T3 action is also critical for the formation of bone. This is particularly true during development, but may also be maintained later in life and obscured by effects on pathways that become amplified as the individual matures and ages. This duality of effects is probably not unique to T3. A number of agents initially identified as growth factors, are now recognized as having actions to promote bone resorption. Parathyroid hormone is now recognized to have important anabolic activity in addition to its well-recognized catabolic effects. Thus, understanding thyroid hormone effects on bone may help us to understand the actions of a number of other factors on the skeleton as well as providing elucidation of the effects of this clinically and pharmacologically important factor itself.

An important insight into how these dual effects could arise derived from the observations that T3 can increase the production of local factors in bone, and that these factors included both important bone growth factors and critical mediators of osteoclastogenesis. These preliminary data indicated that T3 increased the production of the bone growth factor insulin-like growth factor-I (IGF-I) and promoted the production of the inflammatory osteoclastogenic cytokine interleukin-6 (IL-6). It was our hypothesis that these factors were critical for the anabolic and catabolic effects of T3 on bone, respectively. Testing this hypothesis this hypothesis defined the part of the **scope** of the research. Specific Aims IB and IIB were designed to determine this. The other aspect of the research was to elucidate the mechanisms by which thyroid hormone regulates or modulates the production of the local factors (Specific Aims IA and IIA). These studies could provide a framework for understanding how thyroid hormones would affect bone in a given individual and how these effects might be modulated.

BODY

The Specific Aims and the Annual Goals of the Statement of Work were as follows:

IA: Determine the mechanism of the effect of T3 to increase IGF-I in osteoblastic cells
IB: Determine the biological significance of the increased IGF-I for the anabolic effects of T3
IIA: Determine the mechanism and modulation of T3 potentiation of IL-6 production
IIB: Determine the biological significance of the T3 potentiation of IL-6 production

Year 1:

(IA) Optimize conditions for studies of T3 stimulation of IGF-I secretion in osteoblastic cells; begin measurement of IGF-I mRNA by RT-PCR in osteoblastic cells; begin IGF-I promoter analysis

(IB) Produce antisense oligonucleotides to IGF-IR; begin to test effects of excess IGFBP-3 on anabolic effects of T3

(IIA) Select optimal human bone cell model for further studies of T3-enhanced IL-6 production; begin measurement of IL-6 mRNA by RT-PCR (IIA)
(IIB) Optimize conditions for studies of interactions of T3 and IL-1 on resorptive parameters

Year 2:
(IA) Begin studies on T3 effects on the synthesis of IGF-I protein; continue analyses of T3 effects on IGF-I mRNA; begin studies of IGF-I mRNA half-life/rate; continue IGF-I promoter analysis
(IB) Determine effects of IGF-IR antisense oligonucleotides on effects of T3; complete studies on effects of excess IGFBP-3 on anabolic effects of T3; begin studies of antisense IGF-IR cDNA on T3 actions
(IIA) Continue studies of IL-6 mRNA; begin studies on IL-6 half-life/rate; begin IL-6 promoter analysis
(IIB) Begin studies of effects of IL-6 neutralizing antibodies on resorptive actions of T3

Year 3:
(IA) Complete studies on T3 effects on synthesis of IGF-I protein; complete studies on T3 effects on IGF-I mRNA; complete studies on T3 effects on IGF-I mRNA half-life/rate; IGF-I promoter analysis
(IB) Complete studies of antisense IGF-IR cDNA on T3 effects
(IIA) Complete studies of IL-6 mRNA half-life/rate: complete IL-6 promoter analysis; compare effects of T3 with those of other hormones that affect IL-6; investigate interactions of T3 with other resorptive hormones and cytokines on IL-6 production
(IIB) Complete studies of IL-6 and IL-6R neutralizing antibodies on T3 effects; investigate interactions of T3 with other hormones and cytokines on resorptive paramenters

Also, there have been some serendipitous findings from other studies that impact on the overall goal of the understanding the actions of thyroid hormones on bone, and these are included at appropriate points in the report.

Specific Aim IA:

Specific Aim IA was designed to follow up on our published studies (1) showing that T3 increased IGF-I in bone organ cultures and osteoblastic cells. The Specific Aim was to determine the mechanism of the effect. The studies related to this Specific Aim have progressed the more slowly than other components of the research, although new information has been obtained. As reported in the 1st year Progress Report we demonstrated specific binding of ^{125}I-T3 to the human osteoblastic cell lines HOS and MG63 and provided evidence for interaction with both α and β isoforms. In other studies, we have documented binding in ROS 17/2.8 and UMR-106 rat osteoblastic cells and MC3T3-E1 osteoblastic cells.

In UMR-106 cells and Saos-2 cells we found only small and inconsistent changes in IGF-I mRNA in response to T3. Data from the Saos-2 cells using RT-PCR was provided in the year 1 Progress Report. During the current year we have carried out studies using Northern blotting. Osteoblast cultures were treated with thyroid hormone (T3) 0.01 nM - 1 μM for 8, 24 or 48 hr. When total RNA was isolated and analyzed for IGF-I mRNA by Northern blotting using an IGF-I specific riboprobe, no more than a 2-fold increase over untreated cultures was observed. In RT-

PCR analyses in ROS 17/2.8 and human cells a small effect of the same magnitude was also seen. There was no clear dose-dependence of the effects. We are still pursuing this question, and a different approach will be undertaken in continuing studies to determine the extent to which IGF-I production may be transcriptionally regulated (we have requested and obtained a no-cost extension on the grant). Osteoblastic cells will be transiently transfected with IGF-I promoter-luciferase gene fusion constructs. Following treatment with T3, luciferase activity will be measure d as an indication if IGF-I promoter activity. Gene fusions will include human IGF-I promoter regions from -4770, -1208, -1033, -834, -506, -184, and -126 base pairs with respect to the ATG translation start site (these are being kindly provided by T. Owen, Pfizer Central Research, Groton, CT). These studies will initally be carried out in the UMR-106 rat osteoblastic cells on the basis of our success in transfecting these cells and determining IL-6 promoter expression (see Specific Aim IIA, below). As described in the 1st year progress report, T3 downregulated IGF-I receptors in MC3T3-E1, ROS 17/2.8 and UMR-106 cells, indicating that IGF-I mediated anabolic effects of T3 (as documented in the studies carried out under Specific Aim IB, below) are mediated through an increase in IGF-I production, rather than through effects to increase IGF-I binding, and that the approaches we initially proposed and are continuing to carry out on Specific Aim IA are appropriate and important.

Specific Aim IB:

Specific Aim IB was designed to determine the role of IGF-I in the anabolic effects of T3. We have carried out all of the studies outlined in the Statement of Work related to Specific Aim IB. Data from these studies was presented in the first and second year Progress Reports. Several abstracts have been published describing aspects of the work (2-4), and a manuscript (5) is in press in the Journal of Bone and Mineral Research, which is scheduled to appear in the February 2000 issue. Copies of all of these materials are included in the Appendix to this report. We have used three independent approaches to inhibit the action of IGF-I and have shown that these approaches inhibit the proliferative and phenotypic anabolic effects (osteocalcin, alkaline phosphatase, proline incorporation) of T3 on osteoblasts. The approaches used to prevent IGF-I effects were an antibody (αIR3) to the IGF-I receptor, an antagonist peptide (JB1), and an antisense oligonucleotide directed at the IGF-I. A control antibody had no effect. It was proposed to use an excess of IGF binding protein, however, the preliminary experiments utilizing this approach indicated that the amounts that would be required made the costs prohibitive. An interesting observation was that although IGF-I stimulated cell proliferation, the growth factor alone was ineffective in stimulating the effects on the phenotypic anabolic parameters. This appears inconsistent with findings from previous studies on IGF-I (6-9). One important difference between our studies and the previous work was that our medium contained serum that had been stripped with Dowex AF-1-X-10 to remove thyroid hormones. It appears likely from our results that this procedure removes other factors that are required for the expression of the IGF-I effect and that these other factors are stimulated by thyroid hormone treatment.

Our findings from the studies under Specific Aim IB indicate that IGF-I is critical for the anabolic effects of T3, and suggest that an age-related decline in this growth factor could be an important switch to suppress the anabolic effects of the hormone and allow the expression of the catabolic actions.

Specific Aim IIA:

Specific Aim IIA was designed to determine the mechanism by which T3 potentiated the effects of IL-1 to increase IL-6 in bone organ cultures (10), a finding from our laboratory that constituted a preliminary result leading to this proposal. In studies reported in the Progress Report from the first year of the grant, we showed that although potentiation could be demonstrated in some cell lines, this was not a universal effect in all cell lines, although effects in the MG-63 cells reflected a potentiation, which was also seen when IL-6 mRNA was assessed by RT-PCR. In the first year Progress Report we showed that T3 did not increase IL-1 receptor expression in the MG-63 cells, but rather decreased the expression of the receptor. This indicated that the potentiating effects of T3 on IL-1 stimulated IL-6 secretion were not mediated at the level of the expression of the IL-1 receptor.

During the past year, we have have extended our studies of the mechanism by analyzing the actions and interactions of T3 and IL-1 on the IL-6 promoter. Constructs representing the full-length IL-6 promoter (-779) and shorter sequences (-224. -158, -109) coupled with a luciferase reporter were transfected into UMR-106 cells using Lipofectamine Plus®. Other cell lines (MC3T3, ROS 17/2.8, TSA) and other transfection reagents (soybean liposomes, calcium phosphate, DOTAP, Superfect®, Lipofectamine®) were tested, but not found to be satisfactory. A recent experiment indicates that Lipofectamine 2000® may be effective. Figure 1 illustrates the response elements in the IL-6 promoter. IL-1 had stimulatory effects in each of the constructs tested (Figure 2, Figure 3), the greatest effects (Figures 2a, 3a) and a greater effect in combination with T3 (Figure 3a) was found with the shortest promoter construct tested, which contains the NF-κB response element. In other studies with the -224 promoter (Figures 4 and 5), interactions between T3 and and another stimulator of resorption, PTH were examined. T3 and PTH effects were additive or greater. Estrogen decreased the effects of T3, PTH and T3 +PTH, however in the presence of T3, estrogen had less of an inhibitory effect on the response to PTH (Figure 5b). Figure 6 shows effects obtained in a study in which RT-PCR was used to examine effects of IL-1 and T3 on IL-6 mRNA expression in MC3T3-E1 cells. Stimulatory effects of both T3 and IL-1 were observed, although there did not appear to be potentiation.

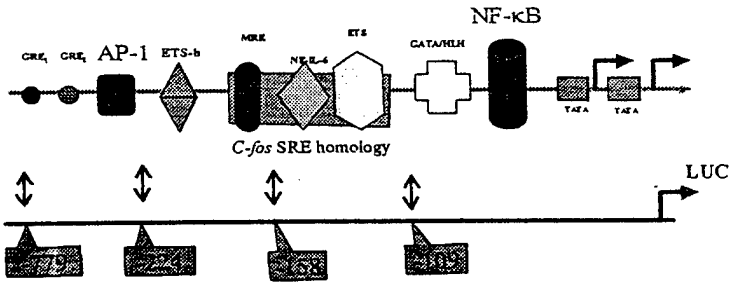
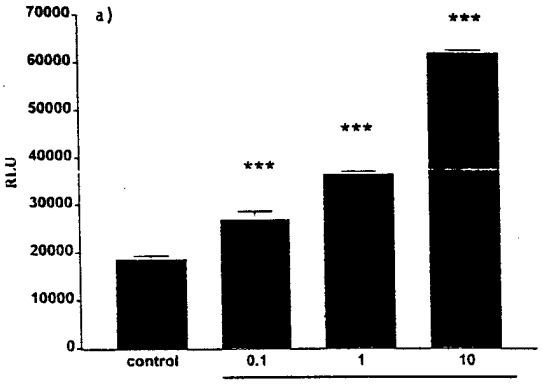
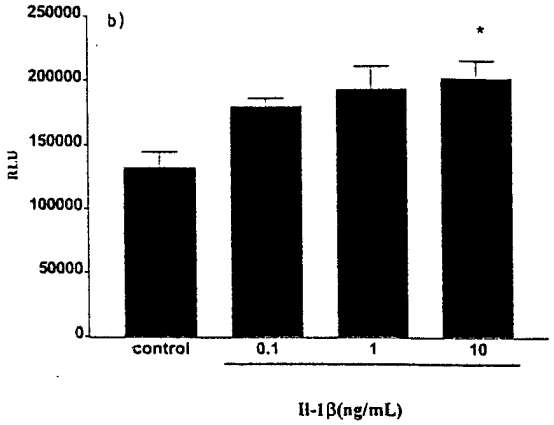


Figure 1. IL-6 promoter constructs used in the present studies.

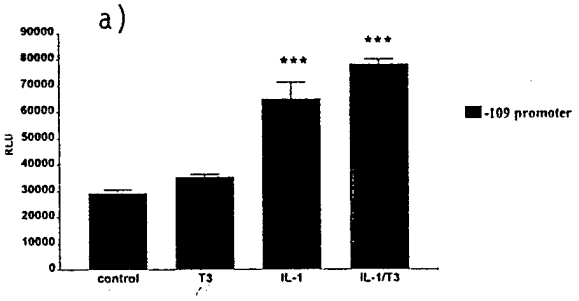


The effect of IL-1 on IL-6 expression with the -109 promoter

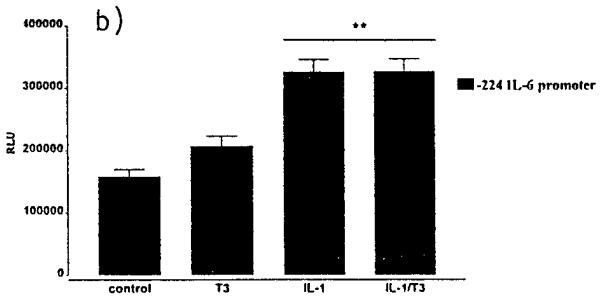


The effect of IL-1 on IL-6 expression with the -224 promoter

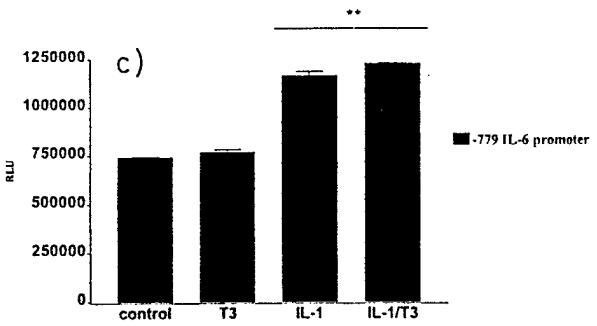
Figure 2. Dose-dependence of effects of IL-1 on a) –109 and b) –224 IL-6 promoter contruct expression; * = $p<0.05$, *** = $p<0.001$ vs. controls.



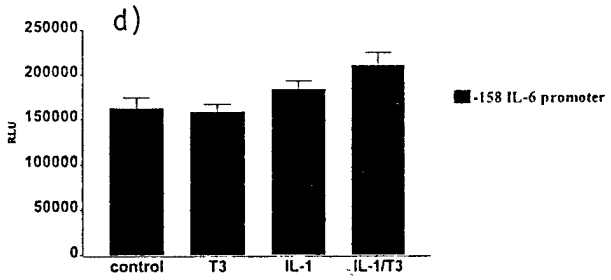
The effect of T3 (10^{-8} M) and IL-1β (10 ng/mL) on the expression of IL-6 -109 promoter



The effect of T3 (10^{-8} M) and IL-1β (10 ng/mL) on the expression of IL-6 -224 promoter



The effect of T3 (10^{-8} M) and IL-1β (10 ng/mL) on the expression of IL-6 -779 promoter



The effect of T3 (10^{-8} M) and IL-1β (10 ng/mL) on the expression of IL-6 -158 promoter

Figure 3. Effects of T3 and IL-1 on a) –109, b) –224, c) –779 and d) –158 IL-6 promoter construct expression; ** = $p<0.01$, *** = $p<0.001$ vs. controls.

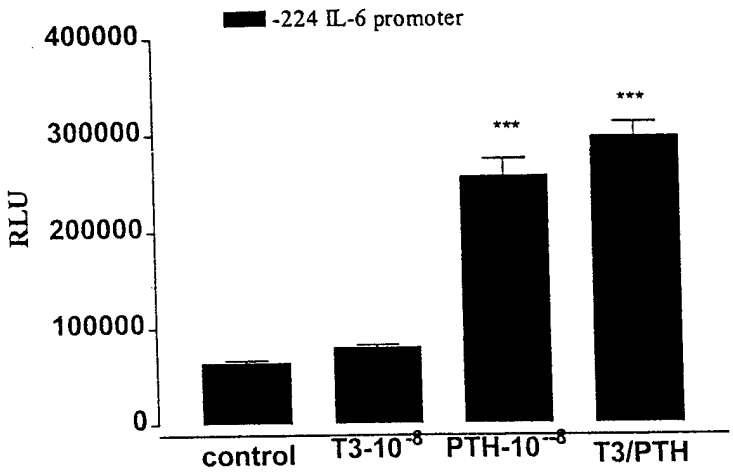


Figure 4. Effects of T3 and PTH on –224 IL-6 promoter construct expression; *** = p<0.001 vs. control.

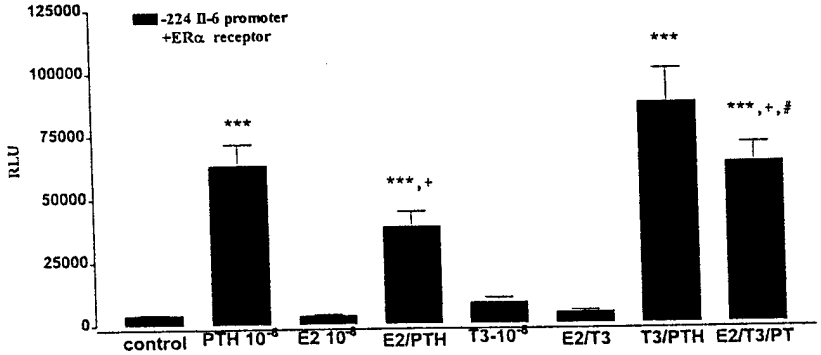
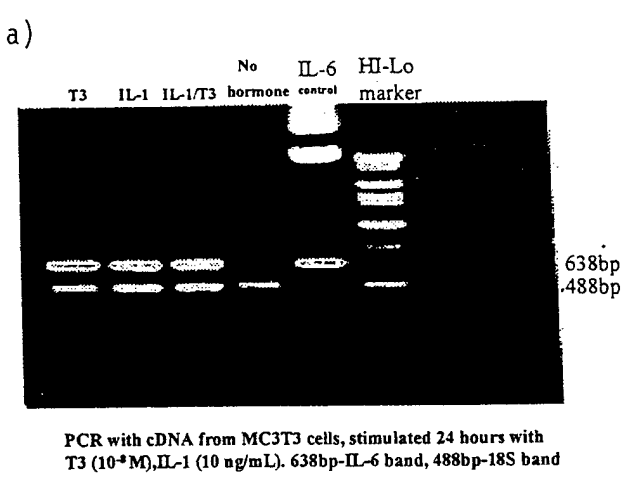


Figure 5. Effects of T3, PTH and estrogen (E2) on –224 IL-6 promoter construct expression; *** = p<0.001 vs. controls, + = p<0.05 vs. treatment without estrogen, # = p<0.05 vs. treatment without T3.



PCR with cDNA from MC3T3 cells, stimulated 24 hours with T3 (10^{-8} M),IL-1 (10 ng/mL). 638bp-IL-6 band, 488bp-18S band

RT-PCR, mRNA from MC3T3 cells treated with T3 (10^{-8} M) and IL-1 (10 ng/mL) until 24 hours . IL-6: 638 bp, 18S: 488bp

Figure 6. a)PCR of cDNA, b)RT-PCR of mRNA from MC3T3–E1 cells treated with T3 and IL-1.

Specific Aim IIB:

Specific Aim IIB was designed to test the hypothesis that the effect of T3 to potentiate IL-6 production is important for the bone-resorbing effects of T3. In studies reported in the first year Progress Report we showed that an antibody to the IL-6 receptor attenuated the resorptive effects of T3. These results were reported and are included in an abstract (2). Other proposed organ culture studies have not yet been completed, including studies of T3 interactions with other hormones and cytokines and studies on enzymatic activities. We attempted to measure the proteolytic activity in the media from the organ cultures by a new fluorometic assay (EnzChek®, Molecular Probes), but the method proved to be insufficiently sensitive for the culture media, although satisfactory standard curves were obtained.

To extend the studies to T3 interactions to other resorptive agents we have carried out studies on parathryoid hormone. Interestingly, we observed that 72 hr pretreatment with PTH or T3 mutually up-regulated each other's receptors in osteoblastic cells (Figures 7), and that pretreatment with T3 enhanced the calcium signals elicited by PTH (Figure 8). As described above (Specific Aim IIA), T3 enhanced PTH effects on IL-6 production. This suggests that the T3/PTH interaction, like the T3/IL-1 interaction, may be another mechanism by which thyroid hormone promotes bone loss. In other studies, T3 did not increase PTH stimulation of cAMP. Since the cAMP signaling pathway has been associated with anabolic effects of PTH, and the calcium signaling pathway may be more important for the catabolic effect, T3/PTH interactions, like T3/IL-1 interactions could also enhance the effects of thyroid hormones on bone loss.

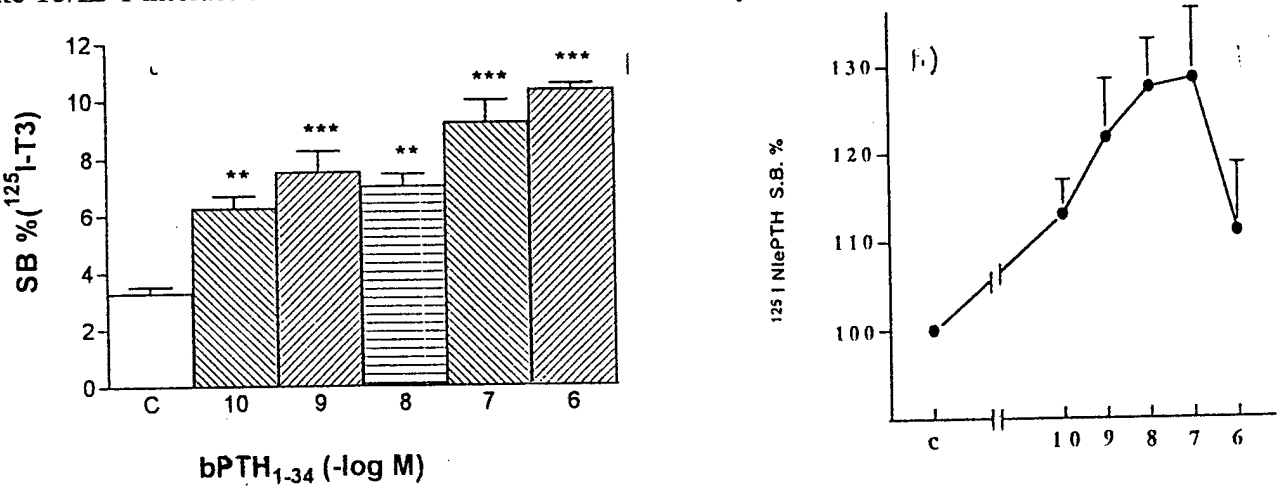


Figure 7. Mutual upregulation of a) T3 and b) PTH receptors in ROS 17/2.8 cells.

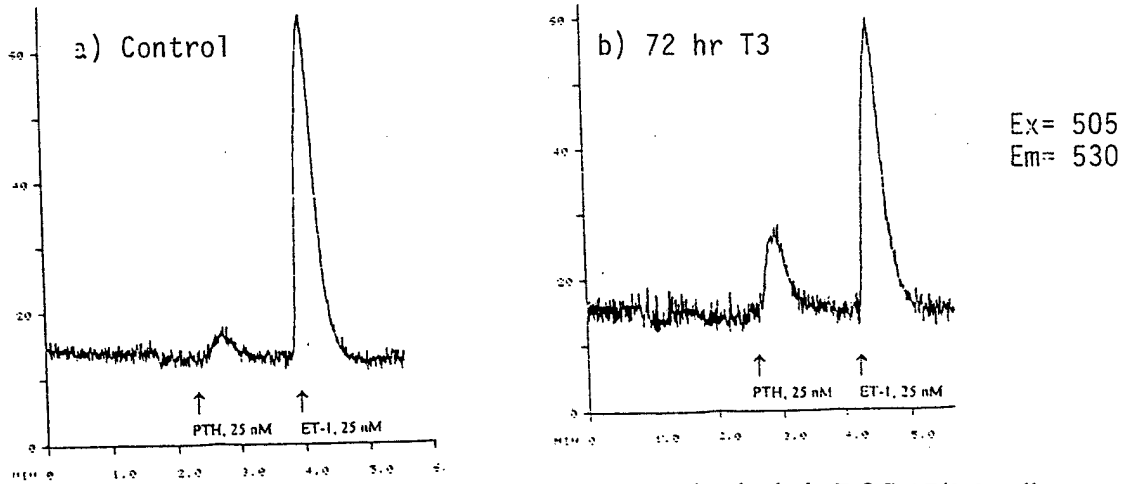


Figure 8. a) vs. b) T3 pretreatment enhances PTH signaling selectively in ROS 17/2.8 cells.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration of the critical role of IGF-I in the anabolic effects of thyroid hormone on bone.
- Demonstration that the effect of thyroid hormone to increase IGF-I production, rather than an effect on IGF-I binding, is likely to be critical for the anabolic effect, since IGF-I binding was decreased by T3.
- Demonstration that IL-6 is important in the effect of thyroid hormone to increase bone resorption
- Demonstration that thyroid hormone can augment IL-1 effects on IL-6 promoter expression, when a construct that represents the NF-κB response element is the dominant regulatory element present

REPORTABLE OUTCOMES

Manuscript:

Huang BK, Golden LA, Tarjan G, Madison LD, Stern PH Insulin-Like Growth Factor Production is Essential for Anabolic Effects of Thyroid Hormone in Osteoblasts. J Bone Miner Res, in press (February 2000).

Abstract (Oral Presentation):

Stern, P.H., Huang, B.K., Du, G-g., Golden, L., Madison, L. Mechanisms of thyroid hormone action on bone. International Conference on Progress in Bone and Mineral Research, 1998.

Abstracts (Posters):

Huang, B.K., Golden, L.A., Stern, P.H. Insulin-like growth factor as a possible mediator of thyroid hormone action in osteoblasts. International Conference on Progress in Bone and Mineral Research, 1998.

Huang, B., Golden, L.A., Madison, L.D., Stern, P.H. Insulin-like growth factor-I (IGF-I) mediates thyroid hormone anabolic actions in osteoblasts. ASBMR-IBMS Second Joint Meeting, 1998.

Gu, W., Stern, P.H., Du, G. Up-regulation by parathyroid hormone of thyroid hormone receptor binding in osteoblast-like ROS 17/2.8 cells. ASBMR-IBMS Second Joint Meeting, 1998.

Training:

Bill K. Huang, carried out work demonstrating the role of IGF-I in the anabolic effects of thyroid hormone in osteoblasts as a Howard Hughes Medical Student Fellow.

CONCLUSIONS

The studies support the hypotheses that the anabolic and catabolic effects of T3 on bone require local cytokines and growth factors. Specifically, IGF-I is critical for the anabolic effects of thyroid hormones to increase osteoblast proliferation and to stimulate the differentiated activities of the osteoblasts, and IL-6 is important in the actions of T3 to stimulate bone resorption. Future studies should be designed to determine whether these are the only local factors required for these actions of T3. The catabolic effects were shown to require a basal effect of IL-1, which was then amplified by T3. Future studies should determine whether thyroid hormone can potentiate the effects of other osteolytic agents. At the time the studies were proposed, much less was understood about the mechanisms of osteoclastogenesis and osteolysis than are known now. Future studies should investigate the effects of T3 on ODF (RANKL) expression in osteoblasts and whether T3 can enhance the effects of ODF. Also, the studies should be extended to in vivo models. In the studies on the mechanism of the effects of T3, evidence has been obtained supporting a hypothesis that the effects of thyroid hormone to increase the production of IGF-I and IL-1 is mediated at the level of mRNA expression. Further ongoing studies will further examine the mechanism of these effects. It will be particularly germane to determine whether physiological changes, including aging result in changes in the relative production of the of the local factors or of the sensitivity of bone to the local factors. Such findings could help to explain the differences in the effects of T3 during growth and during maturity and aging.

In summary, the significance of the work is that it has revealed pathways by which T3 can have either beneficial or deleterious effects on bone, these being the involvement of IGF-I in the anabolic effects and of IL-6 in the resorptive actions. Further, the studies have provided evidence for the mechanisms of the effects of T3 on the expression of these local bone factors. These findings help us to understand how disorders of the thyroid and supplemental thyroid hormones can influence bone integrity. They may provide new insights into sites at which therapy of bone disorders can be targeted.

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APPENDICES

Abstracts from work

Stern, P.H., Huang, B.K., Du, G-g., Golden, L., Madison, L. Mechanisms of thyroid hormone action on bone. International Conference on Progress in Bone and Mineral Research, 1998. Results included under Specific Aim IB, IIB

Huang, B.K., Golden, L.A., Stern, P.H. Insulin-like growth factor as a possible mediator of thyroid hormone action in osteoblasts. International Conference on Progress in Bone and Mineral Research, 1998. Results included under Specific Aim IB

Huang, B., Golden, L.A., Madison, L.D., Stern, P.H. Insulin-like growth factor-I (IGF-I) mediates thyroid hormone anabolic actions in osteoblasts. ASBMR-IBMS Second Joint Meeting, 1998. Results included under Specific Aim IB

Gu, W., Stern, P.H., Du, G. Up-regulation by parathyroid hormone of thyroid hormone receptor binding in osteoblast-like ROS 17/2.8 cells. ASBMR-IBMS Second Joint Meeting, 1998. Results included under Specific Aim IIB

Manuscript in press:

Huang BK, Golden LA, Tarjan G, Madison LD, Stern PH Insulin-Like Growth Factor Production is Essential for Anabolic Effects of Thyroid Hormone in Osteoblasts. J Bone Miner Res, in press (February 2000).

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MECHANISMS OF THYROID HORMONE ACTION ON BONE

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Thyroid hormone plays an important role in the normal development and mineralization of the skeleton. Thyroid hormone excess, especially in the adult, can result in loss of bone and predisposition to fractures. Since thyroid hormone regulates expression of multiple genes, many factors could potentially contribute to or modulate the effects of thyroid hormone on bone. Recent studies from our laboratory and other groups provide evidence to support the possible involvement of several diverse mechanisms. Evidence for three mechanisms that could play a role in the effects of thyroid hormone on bone will be presented: 1) Thyroid hormone increases the production of insulin-like growth factor-I in bone organ cultures and osteoblastic cells. This could contribute to anabolic effects of thyroid hormone. In support of this possibility, we find that interference with the action of insulin-like growth factor-I by antibody or antisense oligonucleotides can attenuate anabolic actions of thyroid hormone on osteoblastic cells. 2) Thyroid hormone potentiates the production of interleukin-1-mediated interleukin-6 production in bone organ cultures. The increase in interleukin-6, an osteoclastogenic factor, could contribute to the catabolic effects of thyroid hormone. In support of this possibility, we find that an antibody to the interleukin-6 receptor can decrease the resorptive effects of thyroid hormone in bone organ culture. 3) Thyroid hormone stimulates the expression of receptors to parathyroid hormone in osteoblastic cells. This could conceivably contribute to either the anabolic or catabolic effects of thyroid hormone by influencing the response to circulating parathyroid hormone and/or locally released parathyroid hormone-related peptide. Factors that influence these processes, including age-related changes in their sensitivity and physiological or exogenous antagonists or promoting factors, could determine the ultimate effect of thyroid hormone on bone in an individual.

Bone 22: 421, 1998

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INSULIN-LIKE GROWTH FACTOR I (IGF-I) AS A POSSIBLE MEDIATOR OF THYROID HORMONE ACTION IN OSTEOBLASTS.

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Thyroid hormone (T3) is a critical regulator of skeletal function, with both anabolic and catabolic effects. Recent studies, including those from our laboratory, suggest that T3 increases the production of IGF-I and the expression of IGF-I mRNA in osteoblasts. IGF-I is an important endogenous regulatory factor in bone with autocrine/paracrine effects. To assess the potential role of IGF-I as a mediator of T3 effects, we characterized phenotypic markers of osteoblast activity in MC3T3-E1 cells and in primary mouse osteoblasts exposed to T3 alone and in the presence of agents that interfere with IGF-I actions. 72 hour treatment with 10 nM T3 significantly increased ^{3}H-proline incorporation, alkaline phosphatase (ALP), and osteocalcin (OCN). However, the same treatment caused a significant decrease in ^{3}H-thymidine uptake. When the cells were simultaneously treated with αIR3, a neutralizing monoclonal antibody to IGF-I receptor, the stimulatory effects of T3 were attenuated. Similar results were also observed when the cells were incubated with phosphothiolated antisense oligonucleotide (AS) directed against the IGF-I receptor gene prior to treatment with T3. Treatments with either αIR3 or AS alone resulted in measurements similar to the corresponding control. Results from experiments with MC3T3-E1 cells and primary osteoblasts are summarized below as % of control ± SD.

	Treatments			
	control	10 nM T3	10 nM T3 + αIR3	10 nM T3 + AS
^{3}H-proline incorporation[1]	100	143±4*	116±7	NA[3]
ALP[1]	100	165±30**	NA	90±10[Y]
OCN[2]	100	627±69**	NA	308±39[Y]
^{3}H-thymidine incorporation[2]	100	60±3**	72±7	NA

[1] MC3T3-E1 cells; [2] Primary osteoblasts; [3] NA – not available
* p<0.01 vs. control; ** p<0.001 vs. control; [Y] p<0.01 vs. 10 nM T3

Our data show 10 nM T3 to have anabolic effects on osteoblasts. T3 seems to promote the cell phenotype toward differentiated osteoblastic functions rather than toward proliferation. IGF-I appears to play an important role in skeletal effects of T3 and may be one of the mediators of T3 action on bone.

*Acknowledgment: This project is funded in part by a Howard Hughes Medical Institute Research Training Fellowship for Medical Students given to Bill K. Huang.

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SA142

Insulin-Like Growth Factor-I (IGF-I) Mediates Thyroid Hormone Anabolic Actions in Osteoblasts. Bill K. Huang,*[1] Laurence A. Golden,*[1] Laird D. Madison,*[2] Paula H. Stern.[1] [1]Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, IL, [2]Endocrinology, Northwestern University Medical School, Chicago, IL.

Thyroid hormone (T3) is a critical regulator of skeletal function, having both anabolic and catabolic effects. Recent studies, including those from our laboratory, indicate that T3 increases the production of insulin-like growth factor-I (IGF-I) and the expression of IGF-I mRNA in osteoblasts. IGF-I is an important endogenous regulatory factor in bone with autocrine/paracrine effects. To assess the potential role of IGF-I as a mediator of T3 actions, we characterized phenotypic markers of osteoblast activity in MC3T3-E1 osteoblastic-like cells and in primary mouse osteoblasts exposed to T3 alone and in the presence of agents that interfere with IGF-I actions. The markers measured included (1) ^{3}H-proline incorporation as a measurement of protein synthesis, (2) alkaline phosphatase activity, and (3) osteocalcin production. We also measured ^{3}H-thymidine uptake to assess proliferative activity. Cells treated with 10 nM T3 for 72 hours had significantly increased ^{3}H-proline incorporation, alkaline phosphatase, and osteocalcin. When the cells were simultaneously treated with αIR3, a neutralizing monoclonal antibody to IGF-I receptor, or JB1, an IGF-I analogue antagonist, the stimulatory effects of T3 were attenuated. Similar results were also observed when the cells were transfected with a phosphothiolated antisense oligonucleotide complementary to the IGF-I receptor gene, prior to treatment with T3. Cells transfected with nonsense oligonucleotides did not show attenuated responses. Treatment with αIR3, JB1, or antisense oligonucleotide alone did not have significant effects. IGF-I alone did not stimulate phenotypic markers of osteoblast activity. However, both IGF-I and T3 produced time-dependent, biphasic effects on mitogenic activity, and the stimulatory effects of T3 on proliferative activity were blocked by antagonists to the IGF-I receptor. In summary, we have shown that anabolic effects of T3 on ^{3}H-proline incorporation, alkaline phosphatase activity, and osteocalcin are attenuated by treatments that interfere with IGF-I actions, indicating that IGF-I plays an important skeletal anabolic effects of T3. Since IGF-I alone failed to stimulate these differentiated functions under the conditions where T3 elicted responses, our results suggest that IGF-I production is necessary but not sufficient for the anabolic effects of T3.

Bone 23: S561, 1998

W221

Up-regulation by Parathyroid Hormone of Thyroid Hormone Receptor Binding in Osteoblast-like ROS17/2.8 Cells. Wen-Xia Gu,[2] Paula H. Stern,[1] Guo-Guang Du.[1] [1]Molecular Pharmacology & Biological Chemistry, Northwestern University, Chicago, IL. [2]Center for Endocrinology, Metabolism & Molecular Medicine, Northwestern University, Chicago, IL.

Parathyroid hormone (PTH) and thyroid hormone (T3) both play important roles in bone remodeling. There may be interaction between T3 and PTH; results from two patients with concomitant Graves' disease and primary hyperparathyroidism suggested that T3 potentiated the osteoclastic effect of PTH (Arem et al,1986). We have previously found that PTH receptor binding and PTH receptor mRNA expression were increased following T3/T4 treatment in ROS 17/2.8 cells. In the present study we examined the effect of PTH on thyroid hormone receptor (TR) binding in osteoblast-like ROS 17/2.8 cells. Confluent cells were serum-depleted for 24 h. Cells were supplemented with 0.1% FBS and treated with PTH (10^{-11}- 10^{-7} M) or PTHrP (10^{-9} M) for 3-4 days. Medium was changed every day. Nuclear extracts were prepared and the protein contents were standardized. Extracts were incubated overnight at 4°C with ^{125}I-l-liothyronine (0.25nM). Free and bound ^{125}I were separated by vacuum filtration through membranes (Millipore HA,0.45μm), and the radioactivity on the membrane was measured. Specific binding was calculated by subtraction of the non-specific binding (binding in the presence of non-labeled T3, 10^{-7} M). The results showed that both PTH and PTHrP stimulated TR binding with maximum effects of PTH observed at 10^{-9} - 10^{-8} M. The specific binding gradually increased over 4 days of PTH treatment. These findings indicate that TR can be up-regulated by PTH. Taken together with our previous findings that T3 can up-regulate PTH receptors, it appears that the effects of PTH and T3 can be strengthened by mutual up-regulation of their receptors.

Bone 23: S360, 1998

Title: Insulin-Like Growth Factor-I Production is Essential for Anabolic Effects of Thyroid Hormone in Osteoblasts*

Running Title: Insulin-Like Growth Factor-I and Thyroid Hormone Effects in Osteoblasts

*Part of this work was presented at the International Conference on Bone and Mineral Metabolism in Vienna, Austria in April, 1998 and the ASBMR-IBMS 2nd Joint Meeting in San Francisco, CA in December, 1998.

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ABSTRACT

Thyroid hormone (T3) and insulin-like growth factor-I (IGF-I) are critical regulators of skeletal function. T3 increases IGF-I production in bone. To assess the potential role of IGF-I as a mediator of T3 actions, we characterized phenotypic markers of osteoblast activity in two osteoblast models, normal mouse osteoblasts and MC3T3-E1 cells, exposed to T3 alone or under conditions that interfere with IGF-I actions. T3 significantly increased osteoblast ^{3}H-proline incorporation, alkaline phosphatase, and osteocalcin. Both αIR3, a neutralizing monoclonal antibody to the IGF-I receptor, and JB1, an IGF-I analogue antagonist, attenuated the stimulatory effects of T3. T3 effects were also decreased in cells transfected with antisense oligonucleotide to the IGF-I receptor gene. Both IGF-I and T3 had mitogenic effects that were inhibited by the antagonists. IGF-I by itself did not stimulate ^{3}H-proline incorporation, alkaline phosphatase, and osteocalcin in the models used, revealing that although IGF-I is essential for the anabolic effects of T3, it acts in concert with other factors to elicit these phenotypic responses.

Key words: insulin-like growth factor-I, thyroid hormone, bone formation markers, growth factor regulation, osteoblasts

INTRODUCTION

Thyroid hormones (T3/T4) are critical regulators of skeletal development and maturation. Evidence demonstrating effects of T3 to promote both the formation and breakdown of bone can be found in clinical observations as well as in vivo investigations in animals (1,2). Long-standing juvenile hypothyroidism can cause severe growth retardation when thyroid hormones are not replaced (3). T4 treatment in children with congenital hypothyroidism resulted in a positive correlation between bone age and the dose of T4 administered (4). Although in children and in young animals, excess T3 causes enhanced bone growth (5,6), it leads to bone loss in adults (7,8). It is well established that both bone formation and resorption are markedly increased in hyperthyroidism (9). Greater increases in the resorption markers than the formation markers suggest an imbalance between resorption and formation, leading to a net loss of cortical and trabecular bone volume (10).

Despite the clinical importance of the regulatory actions of T3 on bone, little is known about the mechanisms by which this occurs. Recent studies have identified the presence of thyroid hormone receptors in osteoblast-like cells and immortalized osteosarcoma cells and have further demonstrated that these receptors are functional (11,12). The expression of certain thyroid hormone receptor isoforms has been shown in osteoclasts (13). The expression of many genes in a variety of tissues, including bone, is regulated by T3 (14). Specifically, production of several osteoblast phenotypic markers such as alkaline phosphatase, osteocalcin, and collagen are affected by T3 in a number of osteoblastic cells (15,16). Furthermore, there is evidence suggesting that certain effects of T3 on bone remodeling are mediated through the production of

local factors. Factors such as prostaglandins and IL-1 have been implicated as mediators of the resorptive effects of T3 on bone (17,18).

As one of the most abundant growth factors present in bone, IGF-I represents a likely mediator of the anabolic actions of systemic hormones. It is found in the plasma and is also produced in many tissues, where it has both autocrine and paracrine action (19,20). IGF-I stimulates bone production by increasing osteoblast proliferation and matrix synthesis (21,22). T3 causes an increase in IGF-I production in UMR-106 osteoblastic cells and bone organ cultures (23,24). IGF-I mRNA in MC3T3-E1 osteoblastic cells is also increased after T3 treatment (25). The IGF-I gene is regulated by several different hormones. GH is the prototypic regulator of the IGF-I gene, especially in the liver. T3 has been shown to interact with GH and, together, these hormones regulate IGF-I production by the liver and other tissues (26). Levels of growth hormone (GH) and insulin-like growth factor-I (IGF-I) are reduced in hypothyroidism, but treatment with GH alone failed to restore normal growth in hypothyroid children (27). Although the biological significance is still unclear, estrogen can induce IGF-I mRNA in osteoblast cell lines (28). In addition, parathyroid hormone (PTH) also increases IGF-I mRNA in bone cell cultures (29). IGF-I appears to mediate specific anabolic effects of PTH on bone; interference with IGF-I blocks the anabolic actions of PTH (22).

The purpose of this study was to determine whether IGF-I activity is important for the skeletal effects of T3 on bone formation. Since both IGF-I and T3 have anabolic actions on bone, and T3 also increases IGF-I production in bone, it was important to determine whether IGF-I could be a mediator of T3 actions on bone. We used several different complementary approaches to specifically interfere with the IGF-I actions in osteoblasts, and to determine whether these interventions would prevent the anabolic effects of T3.

Osteoblastic functions assessed included total protein synthesis based on proline incorporation and the production of the phenotypic markers of osteoblastic activity, osteocalcin and alkaline phosphatase. We also measured the mitogenic activity of the osteoblasts. Normal mouse osteoblasts and/or MC3T3-E1 cells, a widely used clonal osteoblast cell line, were used for the studies.

Our data show that interference with IGF-I actions attenuates T3-stimulated osteoblastic functions, and thus support a model in which IGF-I represents a critical factor for the anabolic effects of T3.

METHODS

Cell culture

Primary osteoblast cultures were prepared from calvarial bones obtained from 6-7 day old neonatal mice (30). Mice were housed and sacrificed in accordance with policy at the Northwestern University Animal Care and Use Facility. After the calvarial bones were dissected, cells were released from the bone by 6 sequential 20 min. collagenase digestions. A pool from digestions 2-6 was collected and seeded into 75-cm^2 flasks for culture in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5 % fetal bovine serum (FBS) and 100 ug/ml penicillin and streptomycin. This primary culture was allowed to grow to confluence and was trypsinized for subculture onto appropriate experiment dishes (either 24- or 96- well multi-well plates). This subculture (technically passage 2) expressed a consistent osteoblastic phenotype based on ALP, OCN and ^{3}H-proline incorporation, and was used to study osteoblast responses to agonist stimulation.

Mouse osteoblastic MC3T3-E1 cells were cultured in αMEM supplemented with 5% FBS and 50 ug/ml gentamycin. Cells were passaged every 7 days by harvesting with 0.1 % trypsin-EDTA and re-seeding in 75-cm^2 flasks. For experiments, MC3T3-E1 cells between passage numbers 3 and 14 were seeded into 60-mm dishes or 24- or 96-well cluster dishes. Assessment of mature osteoblastic phenotypic markers such as ALP and OCN in MC3T3-E1 cell was performed in cells after 18-days culture. The long culture time was required for MC3T3-E1 cells to develop into more mature osteoblasts with the ability to express detectable levels of ALP (31). The cells were seeded and grown in normal serum media for 3 days and the cells were then cultured in T3-

free medium for 18-days prior to treatment with agonists. Medium was changed every 3 days.

For experiments assessing mitogenic activity, once the cells reached 60-80 % confluence after seeding, cells were made quiescent by incubation for 18 hours in medium supplemented with 0.1% bovine-serum albumin (BSA) and antibiotic (hereafter designated as serum-free medium). For experiments involving effects of hormonal manipulation on osteoblast functions, cells were cultured in hormone-depleted medium (designated as T3-free medium) after the cells reached confluence in normal medium. Treatment with Dowex AF-1-X-10 resin (Sigma) was used to remove T3 and T4 hormones from serum with reasonable selectivity. This method, originally reported by Samuels et al., reduced T3 from 150 ng/dl to <2.0 ng/dl and T4 from 6.8 ug/dl to 0.08 ug/dl in calf serum (32). T3-free medium contained 5% resin-treated FBS, 50 ug/ml ascorbic acid, and appropriate antibiotics.

^{3}H-thymidine incorporation

Osteoblasts were plated in 24-well cluster dishes with normal medium at 20,000 cells/well. They were made quiescent as described above and then cultured in serum-free medium with either T3 or IGF-I for a pre-determined treatment period. The concentrations of T3 or IGF-I used were the lowest concentrations that caused a significant response and they were determined based on preliminary studies. ^{3}H-thymidine (0.5 uCi/ml) was added to the cells during the last hour of the treatment in the continued presence or absence of the agonists. At the termination of treatment, ^{3}H-thymidine-containing medium was removed. The cells were washed once with ice-cold PBS, once with 10% trichloroacetic acid (TCA), incubated on ice for 15 min with 10% TCA, and after

one wash with ice-cold 95% ethanol, radioactivity was extracted with 1 N NaOH for assay by liquid scintillation.

^{3}H-proline incorporation

Osteoblasts were subcultured onto 24-well cluster dishes with normal medium at 35,000 cells/well. After the culture reached confluence, cells were hormone-depleted by culturing in T3-free medium for 48 hours prior to treatment. Cells were treated for 72 hours unless otherwise indicated. For measurement of proline incorporation, cells were incubated for the last 2 hours of the treatment period with ^{3}H-proline (1uCi/ml). Samples were prepared similarly to the procedures described for ^{3}H-thymidine incorporation. Incorporation of the label into the TCA-precipitable fraction was measured by liquid scintillation counting.

Osteocalcin (OCN)

Osteoblasts were seeded onto 96-well cluster dishes at 16,000 cells/well in normal medium. Confluent cells were then cultured in T3-free medium prior to agonist stimulation of pre-determined length. Media from the final 72 hours of treatment were collected and stored at -20° C until assay for OCN. OCN secreted by the osteoblasts was measured by a commercial radioimmunoassay (Biomedical Technologies). This assay uses goat anti-mouse OCN and mouse ^{125}I-osteocalcin tracer. This assay is specific for mouse OCN and recognizes total OCN. The limit of detection of the assay is 1.56 ng/ml.

Alkaline phosphatase (ALP) activity

Osteoblasts were seeded in 96-well culture cluster dishes at 16,000 cells/well in normal medium. Confluent monolayer cells were then cultured in

T3-free medium prior to agonist stimulation. Cells were treated with agonists for 72 hours prior to ALP measurement. ALP in cell lysates was measured by the production of p-nitrophenol from p-nitrophenyl phosphate using a protocol modified from Schlossman, et al. (33). Briefly, medium was removed from the treated cells and the monolayers were washed with cold PBS. After the PBS was aspirated, 100 ul diethanolamine (50 mM, pH 10.5) was added to disrupt the monolayer and 50 ul of 2.5 nM p-nitrophenyl phosphate in glycine buffer (100 nM glycine, 2 mM $MgCl_2$) was added as substrate. The entire complex was incubated at 37°C for 30 min, and the reaction was stopped by the addition of 0.1 N NaOH. Absorbance was read on a Dynatech MR5000 spectrophotometer at 410 nm. Activity was calculated in reference to a standard curve of p-nitrophenol. To assess the effect of IGF-I receptor down-regulation on either T3- or IGF-I- stimulated ALP activity, MC3T3-E1 cells precultured in T3-free medium for 18-days were transfected with 1.66 ng/well of either antisense oligonucleotide (AS-ODN) or mismatch oligonucleotide (MS-ODN) for 4 hours using the protocol described below. After the transfection period, cells were returned to T3-free medium and subsequently exposed to either IGF-I or T3 for 72 hours. ALP activity was measured at the end of the treatment period.

Antagonists to IGF-I receptor

To determine the effects of IGF-I receptor antagonists on IGF-I- or T3-induced cellular functions, experiments were performed in which the normal mouse osteoblasts or MC3T3-E1 cells were exposed to the agonists alone or the presence of antagonists. αIR3 (Calbiochem), a monoclonal antibody specific against the IGF-I receptor and JB1 (Peninsula Laboratories Inc), a peptide analogue of IGF-I, were used as antagonists. Both agents have

specificity for the IGF-I receptor and neutralizing properties that block the IGF-I signaling pathway (34,35). A mouse myeloma non-specific IgG antibody was also used to confirm specificity in the osteoblast model.

Antisense IGF-I receptor oligonucleotides

The AS-ODN, 5'-CCC TCC TCC GGA GCC-3', is complementary to a sequence around the AUG-site of the mouse IGF-I receptor mRNA sequence; MS-ODN is a scrambled version of AS-ODN, 5'-CCC GGA TCC TCC GCC-3'. These sequences were kindly provided by Dr. Sergei Gryaznov (personal communication). To assess the efficacy of the AS-ODN, confluent MC3T3-E1 osteoblasts seeded in 60-mm dishes were transfected with either AS-ODN or MS-ODN (3.22 ug DNA/dish) using Lipofectamine-PLUS reagent following the manufacturer's protocol (Gibco). The cells were transfected in serum-free medium for 4 hours and the medium was then replaced with normal growth medium. Levels of IGF-I receptor in transfected cells were assessed 48-72 hours post-transfection.

Western immunoblot

IGF-I receptor expression was determined by Western immunoblotting using methods described previously (30). Briefly, an aliquot of the whole cell lysates of MC3T3-E1 cells was used for determination of protein using the method of Lowry (36). 15 ug protein was applied per lane, and electrophoresis was performed under denaturing conditions on a 10 % polyacrylamide-SDS gel. After overnight wet transfer to nitrocellulose membranes (Schleicher & Schuell), the blot was probed with a rabbit IGF-I receptor beta-subunit antibody (Santa Cruz) at a 1:500 dilution and followed by subsequent incubation with a goat anti-rabbit, peroxidase-conjugated secondary antibody (Sigma) at a

1:2000 dilution. The blot was visualized by enhanced chemiluminescence (ECL, Amersham) using Kodak X-OMAT LS film. For normalization purpose, the same blot was also probed with a rabbit beta-actin antibody (Sigma). Immune complexes were quantified by densitometry using a Bio-Rad imaging densitometer (BioRad).

Statistical analysis

Data are expressed as mean ± sem. Statistical analysis was carried out by one-way analysis of variance. Significance of differences among means was determined by post-hoc testing, using Tukey's method.

RESULTS

Effects of blockade of IGF-I receptors on mitogenic activity of T3 and IGF-I in osteoblasts

3 nM T3 increased ^{3}H-thymidine incorporation in quiesced normal mouse osteoblasts (figure 1a). This result was also observed with MC3T3-E1 cells treated with 10 nM T3 (figure 2a). As shown, 12 hour T3 treatment increased ^{3}H-thymidine incorporation in normal mouse osteoblasts and MC3T3-E1 cells by 33% and 19% respectively. Treatment with IGF-I significantly increased ^{3}H-thymidine incorporation in both cell models. A 59% increase in ^{3}H-thymidine incorporation was seen in normal osteoblasts treated with 1 nM IGF-I for 12 hours (figure 1b). MC3T3-E1 cells treated with 10 nM IGF-I for 12 hours showed an 82% increase compared to control cells (figure 2b). The concentrations of T3 and IGF-I used were the lowest levels that showed a significant effect during preliminary studies.

When normal mouse osteoblasts or MC3T3-E1 cells were co-incubated with T3 and a neutralizing antibody to the IGF-I receptor (αIR3), the stimulatory effect of T3 on ^{3}H-thymidine incorporation was blocked (figures 1a and 2a). Treatment with T3 in the presence of 1.5 ug/ml of αIR3 attenuated the stimulatory effect of T3 to the level of non-stimulated control cells. The same concentration of αIR3 also blocked the increased ^{3}H-thymidine incorporation stimulated by IGF-I in both cell models (figures 1b and 2b). Treatment with αIR3 alone had no effect on basal proliferative responses. A non-specific, control IgG antibody failed to inhibit either IGF-I or T3-stimulated responses in MC3T3-E1 cells or normal mouse osteoblasts.

Effects of IGF-I receptor antagonists on T3 stimulated osteoblastic functions

^{3}H-Proline Incorporation

The effects of 72 hour T3 treatment on ^{3}H-proline incorporation in confluent normal mouse osteoblasts precultured in T3-free medium are illustrated in figure 3a. 10 nM T3 caused a 41% increase in osteoblast ^{3}H-proline incorporation. In this model and under the conditions used, 10 nM IGF-I elicited no stimulation. There was no synergism in the observed responses when the cells were treated with both T3 and IGF-I (data not shown). When cells were treated with 10 nM T3 and in the presence of either αIR3 or peptide analogue of IGF-I, JB1, the T3-stimulated ^{3}H-proline incorporation was attenuated to the level of control. Although both αIR3 and JB1 appear to have slight stimulatory effects on basal ^{3}H-proline incorporation, these changes were not statistically significant.

10 nM T3 also stimulated ^{3}H-proline incorporation in confluent MC3T3-E1 cells grown in T3-free medium. As shown in figure 3b, 10 nM T3 caused a 43% increase in ^{3}H-proline incorporation. As in normal mouse osteoblasts, no significant stimulation was observed with 10 nM IGF-I treatment. The stimulatory effect of T3 on ^{3}H-proline incorporation was attenuated by αIR3. αIR3 alone did not change ^{3}H-proline incorporation when compared with control.

Osteocalcin (OCN)

For these studies, normal mouse osteoblasts, grown to confluence and precultured in T3-free medium for 48 hours, were incubated with 1 nM T3 for 72 hours. Higher concentrations of T3 did not elicit greater stimulation and we chose the lowest concentration that elicited a significant response for the

antagonist studies. The culture media were collected and assayed for OCN production using a RIA. In media from control cells, OCN was 2.3±2.6 ng/ml (mean ± sem, N=6). As shown in figure 4a, 1 nM T3 induced an approximately 50-fold increase in OCN production by the osteoblasts. Similar to the findings on ^{3}H-proline incorporation, 10 nM IGF-I did not stimulate detectable osteocalcin production under the conditions used. To determine whether IGF-I was required for the T3-stimulated OCN production, the cells were treated with T3 in the presence of the IGF-I receptor antibody, αIR3. αIR3 (0.75 - 3.0 ug/ml) significantly decreased T3-stimulated OCN production. αIR3 alone had no effects on OCN production.

T3 also stimulated OCN production in MC3T3-E1 cells. As shown in figure 4b, the maximal effect was obtained with 10 nM T3. OCN from control MC3T3-E1 cells was 0.72±1.5 ng/ml (extrapolated value, below the detectation limit of the assay) and 10 nMT3 stimulated OCN production to 11±5 ng/ml. Although OCN stimulation in MC3T3-E1 cells treated with T3 was statistically significant compared to that in the control cells, the OCN concentration in MC3T3-E1 cells was significantly less than levels observed in normal osteoblasts. For these reasons, we focused our efforts on the normal osteoblasts and did not study the effects IGF-I antagonists on T3 stimulated OCN production in the MC3T3-E1 cells. As was seen in normal mouse osteoblasts, IGF-I did not increase OCN production in the MC3T3-E1 cells.

Alkaline phosphatase

We attempted to study the effects of T3 on ALP activity normal mouse osteoblasts. As shown in figure 5a, the expression of ALP in these cells did not show any stimulation after 72 hr treatment with T3 or IGF-I. The baseline ALP

level in confluent mouse osteoblasts was four times the baseline ALP level in confluent MC3T3-E1 cells.

A stimulatory effect of T3 on ALP in MC3T3-E1 cells was evident when confluent MC3T3-E1 cells grown for 18 days were treated with 10 nM T3 for 72 hours (total culture time of 21 days). T3-treated cells showed a 34% increase in ALP activity compared with non-treated cells (figure 5b). 10 nM IGF-I treatment did not change ALP levels significantly. Co-treatment with 10 nM IGF and 10 nM T3 did not produce stimulation significantly different from the response to 10 nM T3 alone (data not shown).

The T3 stimulated ALP activity was attenuated in MC3T3-E1 transfected with an AS-ODN that is complementary to mouse IGF-I receptor mRNA (figure 6). In these cells, T3 failed to elicit a significant increase in ALP. Cells transfected with 1.66 ug/ml of the mismatch oligonucleotides did not cause an attenuated response to T3. Western immunoblotting for IGF-I receptor in MC3T3-E1 cells confirmed the specificity of AS-ODN for the IGF-I receptor. As shown in figure 6, MC3T3-E1 cells transfected with 1.66 ug/ml AS-ODN showed a 40% decrease in IGF-I receptor expression. Cells transfected with same amount of MS-ODN did not show decreased receptor expression. Transfection of the MC3T3-E1 cells with either AS-ODN or MS-ODN had no effect on the levels of actin protein expression.

DISCUSSION

The importance of T3 to maintain normal skeletal physiology is well accepted. However, the mechanism of T3 effects on bone remodeling remains to be elucidated. We focused our project specifically on studying the anabolic actions of T3 on bone. These anabolic responses included osteoblast proliferation and osteoblast phenotypic markers such as ALP activity, OCN production, and collagen synthesis. An improved understanding of this basic anabolic process elicited by T3 could be beneficial in the management of skeletal problems in patients with altered thyroid status either due to disease processes or of iatrogenic origins, and is thus of clinical importance.

Results from previous studies that showed T3 stimulates IGF-I production in bone models suggest the possibility that IGF-I could be responsible for some of the anabolic effects of T3 in bone tissues. Both T3 and IGF-I have skeletal anabolic effects in vivo: hypothyroid patients, if untreated, often show a bone age delayed more than 2 standard deviations from their chronological age; and with subsequent thyroid replacement therapy, the bone age positively correlates with the concentration of serum T4 (4). A patient with a T3 receptor mutation (homozygous deletion of threonine-337 in TRβ gene) showed similar abnormal skeletal development (37). Similarly, the importance of IGF-I to skeletal systems is evident from the delayed bone development in IGF-I and IGF-I gene knock-out mice; mice with IGF-I receptor mutations show significantly delayed or arrested ossification in the developing skeleton (38). These observations, taken together, further suggest the likelihood that IGF-I may be a candidate to mediate the stimulatory effects of T3 on bone formation or be essential for these stimulatory effects. We therefore undertook to determine

whether blocking IGF-I action could influence T3 anabolic effects in osteoblast cell lines.

Normal osteoblasts and MC3T3-E1 osteoblastic cells were used as model systems for our studies because these cells have the capacity to express phenotypic markers of osteoblast function and to synthesize IGF-I under basal and T3 stimulated conditions (2,39). We selected ^{3}H-proline incorporation, OCN production, and ALP activity because these parameters are generally accepted as osteoblast phenotypic markers. We chose to measure ^{3}H-proline incorporation into total protein and not to distinguish between incorporation into collagenase-digestible protein and noncollagen protein because previous studies have shown that T3 stimulates ^{3}H-proline incorporation into both collagen and noncollagen protein to a similar extent in bone (40).

The choice of the cell type to use for a particular parameter was dictated by both cell characteristics and methodological feasibility. Under our experimental conditions, we were able to adequately measure the base-line ^{3}H-thymidine and ^{3}H-proline incorporation in both normal mouse osteoblasts and MC3T3-E1cells and these parameters were responsive to T3 stimulation. Although OCN production could be detected in both cell models after T3 treatment, the response was much greater in normal osteoblasts. OCN levels in untreated cells were within the working range of the RIA assay in normal osteoblasts only; OCN levels in control MC3T3-E1 cells were below the limit of detection of the assay. Therefore, the effect of the IGF-I antagonist on T3-stimulated OCN production was only studied in normal osteoblasts. Assessment of the effect of the IGF-I antagonist on ALP activity was limited to MC3T3-E1 cells because no stimulatory effects of T3 on ALP were observed in the normal osteoblasts. In previous studies, the effects of T3 on ALP have been variable, with some studies showing stimulatory effects while others reporting

inhibitory or no response. For example, T3 failed to affect ALP activity in a study using UMR-106 cells (41). In normal rat osteoblastic cells, T3 had dose-dependent biphasic effects on ALP (42).

We employed three different independent approaches to specifically interfere with the IGF-I actions in bone. Both the neutralizing antibody, αIR3, and IGF-I peptide analogue, JB1, act as antagonists to the IGF-I receptor to prevent binding of IGF-I and the antisense oligonucleotide prevents the synthesis of IGF-I receptor. Both αIR3 and JB1 were shown to specifically block IGF-I stimulated ^{3}H-thymidine incorporation in various cell lines (34,35). This inhibitory effects of αIR3 was also seen in our cell models where we observed attenuated ^{3}H-thymidine incorporation in response to IGF-I stimulation in cells co-treated with the antagonists. The specificity of effects achieved by αIR3 was further confirmed by the inability of a control IgG antibody to attenuate IGF-I stimulation. A third approach was to inhibit IGF-I action by decreasing IGF-I receptor number using antisense oligonucleotides specific for IGF-I receptor mRNA. Experiments using transfection of oligonucleotides were performed only in MC3T3-E1 cells because we were not successful in transfecting normal osteoblasts. The effectiveness of the AS-ODN to reduce IGF-I receptor protein was demonstrated using Western blotting for the IGF-I receptor. Using multiple approaches to interfere with IGF-I actions made it less likely that the observed responses were results of non-specific side-effects from any one of the antagonists.

Our data demonstrated mitogenic effects of T3. Other studies have shown that T3 has a dose-dependent, biphasic effect on osteoblast growth, with stimulation at low and inhibition at high concentration (43). T3 elicited significant stimulation of ^{3}H-thymidine incorporation in both normal osteoblasts and MC3T3-E1 cells. IGF-I also stimulated ^{3}H-thymidine incorporation in both

models. Consistent with our proposed hypothesis, treatment with neutralizing antibody to IGF-I receptor blocked both IGF-I and T3-stimulated ^{3}H-thymidine incorporation.

Our findings also showed anabolic effects of T3 on the osteoblast systems. T3 treatment caused a significant increase in phenotypic markers of osteoblasts. Normal osteoblasts treated with T3 for 72 hours showed significant increases in both ^{3}H-proline incorporation and osteocalcin production. To determine whether endogenously produced IGF-I was necessary for T3-stimulated osteoblastic responses, we tested the effect of a neutralizing anti-IGF-I receptor antibody on T3 induced ^{3}H-proline incorporation and osteocalcin production. In addition, an IGF-I peptide antagonist, JB1, was tested for its effect on T3-induced ^{3}H-proline incorporation. Both the antibody and peptide antagonist had significant attenuating effects on the T3-stimulated responses in normal osteoblasts, indicating a requirement for IGF-I in T3-induced total protein and osteocalcin synthesis. Treatment with αIR3 antibody also attenuated T3 stimulated ^{3}H-proline incorporation in the MC3T3-E1 cells.

Dependence upon IGF-I was also observed for T3 effects on alkaline phosphatase activity in MC3T3-E1 cells. Treatment with 10 nM T3 greatly stimulated alkaline phosphatase expression, however, cells transfected with AS-ODN did not show increased alkaline phosphatase activity with the same T3 treatment. It is interesting that even a partial reduction in the expression of IGF-I receptor resulted in a significant attenuation in response. Similar results were also reported in a recent study by Muller et al. using ovarian cancer cells transfected with IGF-I receptor antisense ODN (44). They showed that IGF-I receptor protein was reduced by the antisense ODN to 53% of control value and that transfected cells showed decreased proliferation in response to IGF-I stimulation. The control MS-ODN, which we demonstrated by Western

immunoblotting to have no effect on IGF-I receptor expression, did not have an attenuating effect on T3-stimulated alkaline phosphatase activity. This result further confirmed that the IGF-I pathway is necessary for T3-stimulated responses.

Given our results showing that agents that interfere with IGF-I actions can block T3-stimulated mitogenic and anabolic responses in osteoblasts, it was expected that treatment with IGF-I alone would increase both mitogenesis and the osteoblast phenotypic markers. Unexpectedly, in both models, treatment with IGF-I alone caused only a stimulation in mitogenic activity and did not produce a stimulation in ^{3}H-proline incorporation, OCN production, or alkaline phosphatase expression; cells that were co-treated with both IGF-I and T3 did not produce responses that were significantly greater than cells treated with T3 alone. These data were unexpected since previously published studies demonstrated IGF-I to have anabolic effects on bone. IGF-I stimulated both collagen and non-collagen production in cultures of fetal rat calvaria and human osteoblasts (21,45-47).

A major and the most significant difference between our models and those in which IGF-I alone had effects is that others did not use resin-stripped serum, which we used in all of our anabolic studies. It is possible that other factors could have been present and were removed by the stripping procedure; these factors might complement IGF-I to stimulate anabolic actions in bone. This possibility is supported by the findings of Pirskanen et al., who showed that IGF-I alone stimulated mitogenesis in MG-63 cells but failed to stimulate osteocalcin in the absence of other factors such as calcitriol (56). T3 treatment might induce the synthesis of these other factors in its pleiotropic effects on bone.

It may be significant that other studies were performed in different osteoblast systems than those we used. These cell-based differences in response to an IGF stimulus may reflect receptor population and receptor cross-reactivity and depend on cell type species and osteoblast lineage and maturation (48,49). Recent studies have shown differential effects of thyroid hormones on several parameters, including IGF-I, at different skeletal sites (50-52). Malpe et al. further demonstrated differential levels of IGF-I/-II and IGFBPs 3, 4, and 5 at five different skeletal sites (53). Since both of the cell models used in our study were calvarial-derived, it would be of interest in future studies to determine whether the IGF-I dependence of T3 effects observed in the current study are also found in cells from other skeletal sites.

Interactions with various IGF binding proteins (IGFBPs) can also affect the observed responses. Both T3 and IGF-I can increase the production of IGFBPs (24). IGFBPs can modulate IGF actions in bone. Certain IGFBPs inhibit IGF-I actions and others have been shown to have enhancing effects (49,54,55). The relative amounts of the IGFBPs produced by T3 and IGF under different experimental conditions and in different models could play a role in the different responses seen, since the final osteoblast response depends on presence or absence of endogenous IGFs and other IGFBPs.

In summary, different complementary methods of blocking IGF-I actions all attenuated T3-stimulated responses in osteoblasts. We conclude from our findings that IGF-I is a necessary component for T3-stimulated anabolic osteoblast functions and that the stimulation of IGF-I production by T3 is thus essential for the anabolic effects of the hormone. The observation that IGF-I alone did not elicit the same responses as T3 on ^{3}H-proline incorporation, OCN production, or alkaline phosphatase activity (although it stimulated mitogenesis) suggests that IGF-I is a necessary but not sufficient factor for T3 responses.

Other T3-stimulated local factors that are essential for IGF-I action factors may also contribute to the anabolic effects of T3 on bone.

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FIGURE LEGENDS

figure 1

IGF-I and T3 stimulate mitogenic activity in normal mouse osteoblasts and antibody to IGF-I receptor (αIR3) blocks the mitogenic effects of IGF-I and T3. (a) Quiescent normal osteoblasts were treated with 3 nM T3 with or without antibody (1.5 ug/ml αIR3 or 1.5 ug/ml control IgG) alone for 12 hours or in the presence of both antibody and agonist. * * significantly different from control, $p<0.01$. * significantly different from control, $p<0.05$. + + significantly different 3 nM T3, $p<0.01$. (b) Quiescent normal mouse osteoblast were treated 1 nM IGF-I with and without antibody (1.5 ug/ml αIR3 or 1.5 ug/ml control IgG) for 12 hours. * * significantly different from control, $p<0.01$. + + significantly different 1 nM IGF-I, $p<0.01$. ^{3}H-thymidine incorporation reflects value from per well and is expressed as percent control. Data represent mean ± sem, n = 3 or 4 for each treatment group from a single experiment. Experiments were repeated at least three times; each graph is from one representative experiment.

figure 2

Antibody to IGF-I receptor (αIR3) blocks the mitogenic effects of T3 and IGF-I in MC3T3-E1 cells. (a) Quiescent MC3T3-E1cells were treated with 10 nM T3 with or without antibody (1.5 ug/ml αIR3 or 1.5 ug/ml control IgG) for 12 hours. * significantly different from control, $p<0.05$. + significantly different from 10 nM T3, $p<0.05$. (b) Quiescent MC3T3-E1 cells were treated with 10 nM IGF-I with or without antibody (1.5 ug/ml αIR3 or 1.5 ug/ml control IgG) for 12 hours. * * significantly different from control, $p<0.01$. + + significantly different 10 nM IGF-I, $p<0.01$. ^{3}H-thymidine incorporation per well is expressed as percent control. Data represent mean ± sem, n = 3 or 4 for each treatment group from a single

experiment. Experiments were repeated at least three times; each graph is from one representative experiment.

figure 3

Antagonists to IGF-I receptor inhibit T3 stimulate ^{3}H-proline incorporation. (a) Confluent normal mouse osteoblasts were treated with agonists (10 nM T3 or 10 nM IGF-1) for 72 hours with our without either αIR3 (1.5 ug/ml) or JB1 (2 ug/ml). (b) Confluent MC3T3-E1 cells were treated with agonists (10 nM T3 or 10 nM IGF-1) for 72 hours with or without αIR3 (1.5 ug/ml). * * significantly different from control, $p<0.01$. ++ significantly different from 10 nM T3, $p<0.01$. ^{3}H-proline incorporation is determined for each well and data is expressed as percent control. Data represent mean ± sem, n = 4 for each treatment group from single experiment. Experiments were repeated at least three times for each cell type; each graph is from one representative experiment.

figure 4

T3 stimulates OCN production and αIR3 (Ab) blocks the effects of T3 on OCN. (a) Confluent normal osteoblasts were treated with agonists (1nM T3 or 1nM IGF-I) with or without αIR3 antibody (0.75, 1.5, or 3 ug/ml.) (b) Confluent MC3T3-E1 cells were treated with T3 (0.1, 1.0, 10 nM) or 10 nM IGF-I. OCN was measured in the collected conditioned medium and reflects the OCN concentration per well of confluent monolayer. Data represent mean ± sem, n = 6 to 12 for each treatment group. Data were gathered from at least 3 separate experiments and combined for analysis. * * significantly different from control, $p<0.001$; * significantly different from control $p<0.01$; ++ significantly different from 10 nM T3, $p<0.01$.

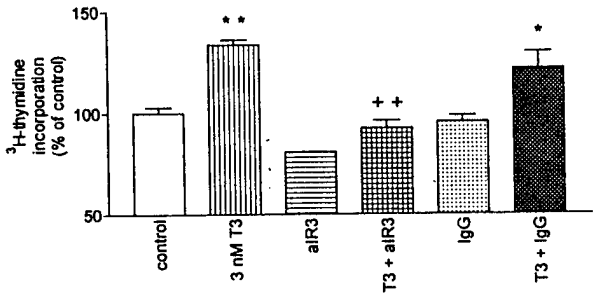
figure 5

Effects T3 treatment on osteoblast ALP activity. (a) Lack of significant stimulation of ALP activity by T3 (0.1, 1.0 and 10 nM) in normal osteoblasts. (b) T3 stimulates ALP activity in MC3T3-E1cells, and IGF-I receptor AS-ODN blocks T3 induced ALP activity. MC3T3-E1 cells were transfected with either AS-ODN or MS-ODN and treated with 10 nM T3 or 10 nM IGF-I for 72 hours. * * significantly different from the respective control, $p<0.01$. + significantly different from non-transfected cells treated with 10 nM T3 , $p<0.05$. ALP activity was measured per well of confluent osteoblast monolayer. Data represent mean ± sem, n = 6 to 12 for each treatment group from a single experiments. Experiments were repeated at least three times for each cell type; each graph is from one representative experiment.

figure 6

IGF-I receptor AS-ODN decreases IGF-I receptor expression in MC3T3-E1 cells. The IGF-I receptor protein level was determined by Western immunoblotting with an anti-IGF-I receptor beta antibody. (a) Levels of IGF-I receptor expression in control (lane 1) MC3T3-E1 cells and in cells transfected with either 1.66 ug/ml AS-ODN (lane 2) or MS-ODN (lane 3) were determined using western immunoblotting. Levels of beta-actin expression were measured and used for normalization. The experiments were repeated at least 3 times; presented graphs are representative of one of experiment. (b) Expression was quantified by densitometry and expressed as the ratio of IGF-I receptor to actin. Data are presented as percent control.

A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^{3}H-thymidine incorporation (% of control)
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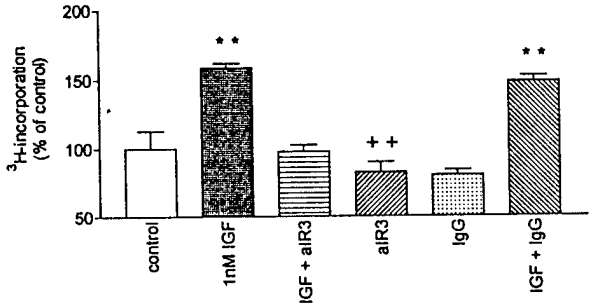
control
3 nM T3
αIR3
T3 + αIR3
IgG
T3 + IgG

B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^{3}H-incorporation (% of control)
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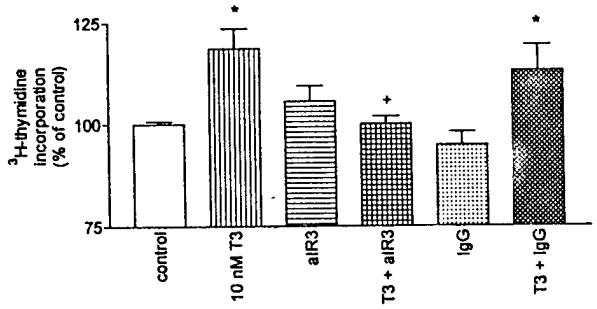
control
1nM IGF
IGF + αIR3
αIR3
IgG
IGF + IgG

A


^{3}H-thymidine incorporation (% of control)
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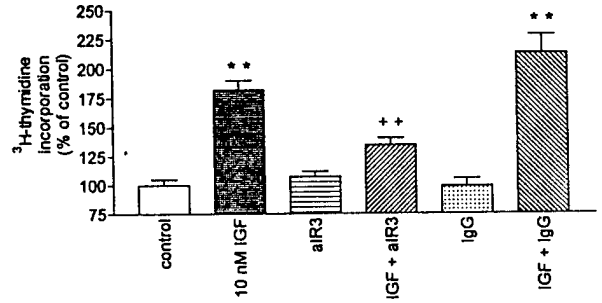
10 nM T3
aIR3
T3 + aIR3
IgG
T3 + IgG

B


^{3}H-thymidine incorporation (% of control)
250
225
200
175
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125
100
75
**
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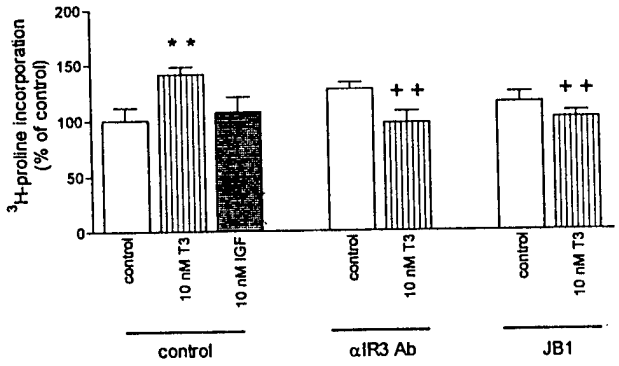
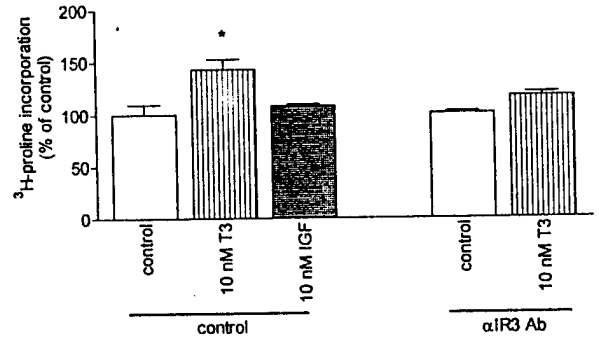
++
control
10 nM IGF
aIR3
IGF + aIR3
IgG
IGF + IgG

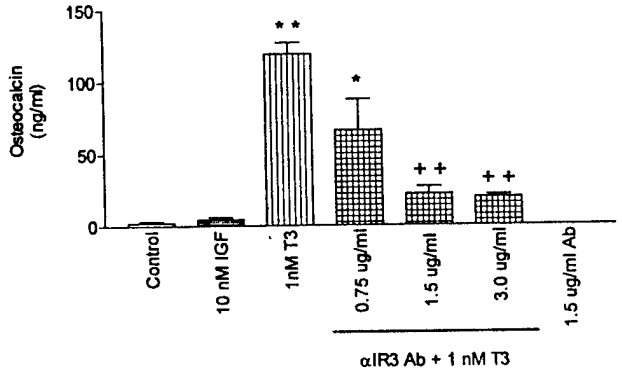
A


^{3}H-proline incorporation (% of control)
200
150
100
50
0
* *
+ +
+ +
control
10 nM T3
10 nM IGF
control
10 nM T3
control
10 nM T3
control
αIR3 Ab
JB1

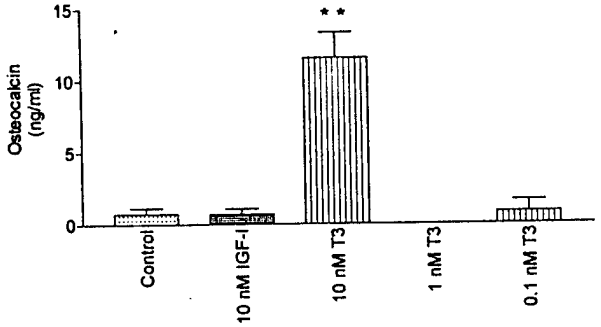
B


^{3}H-proline incorporation (% of control)
200
150
100
50
0
*
control
10 nM T3
10 nM IGF
control
10 nM T3
control
αIR3 Ab

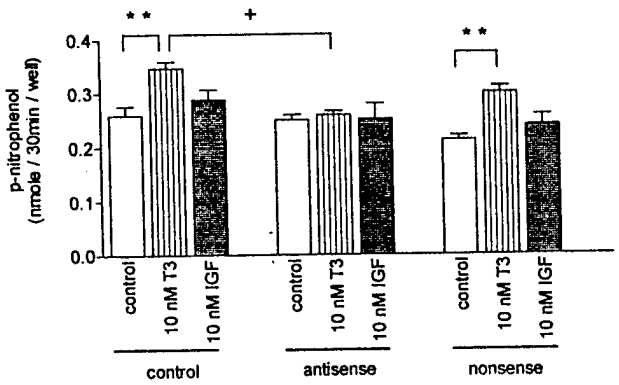
A


Osteocalcin (ng/ml)
150
100
50
0
* *
*
+ +
+ +
Control
10 nM IGF
1nM T3
0.75 ug/ml
1.5 ug/ml
3.0 ug/ml
1.5 ug/ml Ab
αIR3 Ab + 1 nM T3

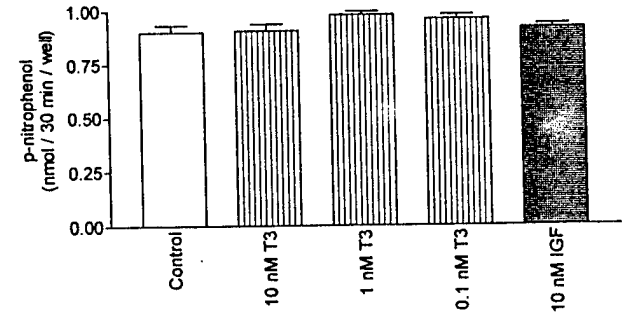
B

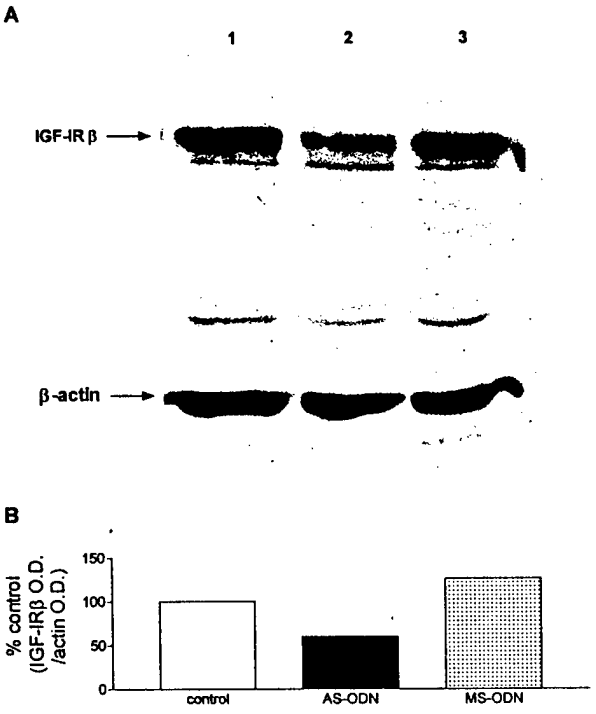

Osteocalcin (ng/ml)
15
10
5
0
* *
Control
10 nM IGF-I
10 nM T3
1 nM T3
0.1 nM T3

A


p-nitrophenol
(nmols / 30min / well)
0.4
0.3
0.2
0.1
0.0
* *
+
* *
control
10 nM T3
10 nM IGF
control
10 nM T3
10 nM IGF
control
10 nM T3
10 nM IGF
control
antisense
nonsense

B


p-nitrophenol
(nmol / 30 min / well)
1.00
0.75
0.50
0.25
0.00
Control
10 nM T3
1 nM T3
0.1 nM T3
10 nM IGF


A
1
2
3
IGF-IR β
β-actin
B
% control
(IGF-IRβ O.D.
/actin O.D.)
150
100
50
0
control
AS-ODN
MS-ODN